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Results from this investigation have provided a basic understanding of how calcium regulates neuronal cytoskeletal function. Calmodulin-dependent protein kinase activity was investigated in cold stable and cold labile microtubule fractions. Calmodulin-dependent kinase was enriched approximately twenty fold over cytosol in cold stable microtubule preparations. Calmodulin-dependent kinase activity in cold stable microtubule preparations phosphorylated microtubule associated protein-2, tubulin, an 80,000 dalton doublet, and several minor phosphoproteins.

This endogenous calmodulin-dependent kinase in cold stable microtubule fractions was identical to CaM-Kinase II isolated from rat brain by several criteria including: 1) subunit molecular weights, 2) subunit isoelectric points, 3) calmodulin binding properties, 4) subunit autophosphorylation, 5) Calmodulin binding subunit composition,

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6) isolation of kinase on calmodulin affinity resin, 7) kinetic parameters, 8) phosphoamino acid phosphorylation sites on tubulin, and, 9) phosphopeptide mapping. Endogenous cold stable calmodulin-dependent kinase activity was isolated from the microtubule fraction by calmodulin affinity resin column chromatography and specifically eluded with EGTA. This kinase fraction contained the calmodulin binding protein and autophosphorylating subunits of CaM Kinase II.

These results indicated that calmodulin-dependent kinase is a major calmodulin binding enzyme system in cold stable microtubule fractions and suggested that it may play a role in mediating some of the effects of calcium on microtubule and cytoskeletal dynamics. Further study of the calmodulin-dependent kinase in microtubule preparations revealed that it was possible to separate an endogenous Calmodulin microtubule associated protein II complex from this fraction. We demonstrated that an endogenous calmodulin-dependent kinase that phosphorylates Map 2 as a major substrate is also present in microtubules prepared under conditions that preserve the kinase.

A fraction containing the calmodulin-dependent kinase and map 2 was separated from the cyclic AMP-dependent kinase map 2 complex by gel filtration chromatography of microtubule protein in high ionic strength buffer. These data demonstrate that both cyclic AMP and calmodulin kinase are present in microtubule preparations and that the may differentially regulate map 2 function by phosphorylating map 2 on two distinct sites. Subsequent studies revealed by peptide mapping that Calmodulin Kinase II phosphorylates map 2 on distinct peptide regions from the cyclic AMP-dependent kinase. These results provide direct evidence that these two kinases may alter map 2 function by phosphorylating different domains of the protein.

Hydrazine is a major component of rocket fuel and other industrial products. Hydrazine can cause acute neurological injury by producing seizures and alterations in neuronal excitability. There is also evidence, however, that hydrazine produces delayed effects on the nervous system producing peripheral neuropathy and delayed neurotoxicity. There is a suggestion that hydrazine may effect the nervous system by altering the neuronal cytoskeleton. Experiments in this study demonstrated that hydrazine in high toxic levels can activate CaM Kinase II activity. This increased activity of CaM Kinase II would be expected to depolymerize the microtubules based on our basic research of the effects of this enzyme on microtubule function. Thus, these results may provide a molecular insight into how hydrazine may destabilize the cytoskeletal network in a neuron. Although it is not possible at this time to prove that this is the cause of hydrazine toxicity to the peripheral nervous system, it is an attractive model.

Experiments are now being designed to determine a more direct correlation between the effects of hydrazine on cytoskeletal dynamics and its long-term toxicity on peripheral nerves. The ultimate goal of these studies would be to develop an understanding of this neuronal toxicity and to develop mechanisms to block its effect pharmacologically. We have specific pharmacological agents that inhibit CaM Kinase II activity. These drugs can prevent the effect of hydrazine on CaM Kinase II in the test tube. If these compounds could block the effects of this toxin on intact neurons, it is possible that it may prevent some of the toxic long-term effects of hydrazine on the nervous system. Further studies may provide important clinical insights into the use of these compounds in blocking delayed hydrazine toxicity.

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           CALMODULIN REGULATED NEURONAL PROCESSES

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THE EFFECTS OF HYDRAZINE AND RELATED COMPOUNDS ON

CALCIUM-CALMODULIN REGULATED NEURONAL PROCESSES

The goals of this project were to develop an experimental system to study the role of calmodulin-dependent protein kinase activity in cytoskeletal preparations and determine the effects of hydrazines on cytoskeletal kinase activity and microtubule polymerization. During the year and a half spent on this grant project, we made considerable progress in accomplishing our objectives.

The laboratory:

1) Prepared CaM Kinase II for study.

2) Identified Calmodulin Binding Proteins in microtubule preparations.

3) Isolated a CaM Kinase II - tubular complex.

4) Demonstrated that CaM Kinase II is associated with microtubules.

5) Characterized the effects of hydrazines on CaM Kinase II.

6) Studied the effects of CaM Kinase II on microtubule polymerization.

7) Correlated the effects of hydrazine on CaM Kinase II and microtubule polymerization.

8) Investigated the effects of pharmacological agents on modulating the effects of hydrazine on CaM Kinase II.

The details of these experiments are presented below. These studies are currently being extended to investigate the effects of hydrazine on isolated membrane currents.

1. **CaM KINASE II PURIFICATION AND CHARACTERIZATION**

The purification to apparent homogeneity and characterization of a brain cytosolic calcium-calmodulin dependent kinase which phophorylates tubulin and microtubule associated proteins as major substrates has now been established. The cytosolic kinase system, purified by sequential chromatography on phosphocellulose resin, calmodulin-affinity resin, and Fractogel TSK HW-55, chromatographs as a homogeneous complex of approximately 600,000 daltons on Sephacryl S-300. This tubulin-associated calmodulin dependent kinase possesses a characteristic group of properties. CaM Kinase II contains two calmodulin binding doublets, rho (52,000 daltons) and sigma (63,000 daltons), with isoelectric points between 6.7 and 7.2. The rho and sigma subunits demonstrate autophosphorylation and show significant homologies as assessed by tryptic peptide fingerprints. CaM Kinase II phosphorylates beta-tubulin equally on threonine and serine residues. Substrate specificity, calmodulin binding properties, subunit composition, and subunit isoelectric points clearly differentiate CaM Kinase II from other previously reported kinases. These publications present the details of our studies on that kinase system. We employed these specific molecular parameters to study CaM Kinase II in microtubule - tubulin systems.

2. **IDENTIFICATION OF CALMODULIN-BINDING PROTEINS IN MICROTUBULE PREPARATIONS AS SUBUNITS OF A CALMODULIN-DEPENDENT PROTEIN KINASE**

Research in this grant project demonstrated that microtubule fractions prepared in vitro contain two major detectable calmodulin-binding proteins. These proteins have been characterized and shown by several criteria to be identical to a purified calcium-calmodulin-

dependent protein kinase which phosphorylated tubulin and microtubule-associated proteins as major substrates. These results indicate that CaM Kinase II is a major calmodulin-binding target enzyme in microtubule preparations and suggest that activation of this kinase system may mediate some of the effects of calcium-calmodulin on microtubule and cytoskeletal dynamics.

a.   Calmodulin-Binding Proteins in Microtubule Preparations

Calmodulin-binding proteins in thrice cycled microtubule preparations were visualized using the technique of Carlin, et al. Figure 1A demonstrates that only two calmodulin-binding proteins were observed with apparent molecular weights of 52,000 and 63,000 daltons. Addition of 2 mM EGTA or excess unlabelled calmodulin abolished all detectable calmodulin-binding. Extended exposure of the calmodulin-binding gels to x-ray film for up to 3 months did not reveal any other higher or lower molecular weight [125I]-calmodulin-binding bands. Resolution of microtubule protein on two-dimensional isoelectric focusing/SDS-PAGE gels demonstrated that the two calmodulin-binding proteins possessed similar isoelectric points near neutrality (Figure 1A). In order to demonstrate the presence of calmodulin-binding proteins associated with microtubule fractions under non-denaturing conditions, microtubule proteins were chromatographed on calmodulin affinity resin (Figure 2). While most of the protein passed through the column into the void volume and the 500 mM salt wash, a protein peak representing less than 2.5% of the original protein was eluted from the column specifically with chelator. This fraction isolated from microtubule preparations by calmodulin affinity chromatography contained only two calmodulin-binding bands of 52,000 and 63,000 daltons as described in Figure 1.

b. **Identification of CaM Kinase II as a Major Calmodulin-Binding Enzyme System in Microtubule Fractions**

Since our work showed that calcium-calmodulin kinase activity is present in microtubule preparations, we investigated the possibility that the calmodulin-dependent kinase activity in microtubule preparations represented the calmodulin-binding proteins in this fraction. Figure 3 demonstrates that endogenous calmodulin-dependent phosphorylation of microtubule protein was observed in microtubule fractions containing the two calmodulin-binding proteins. This endogenous kinase activity phosphorylated microtubule fraction proteins with molecular weights of 50,000, 60,000, 80,000 and 250,000 daltons as well as several other minor phosphoproteins. Phosphorylation of the two calmodulin-binding bands and tubulin represented the endogenous phosphorylation seen in the 50,000 and 60,000 dalton region when analyzed by two-dimensional gel electrophoresis (Figure 4). The fraction adhering to the calmodulin affinity column isolated from microtubules (Figure 2) and containing the two major calmodulin-binding proteins was evaluated for calmodulin kinase activity. This calmodulin column fraction contained calcium-calmodulin-dependent kinase activity, phosphorylating tubulin and MAP-2 as major substrates. The calmodulin-binding bands in this fraction also demonstrated calcium-calmodulin stimulated autophosphorylation when incubated without added substrates.

Tubulin can be separated from microtubule associated proteins by chromatography of microtubule protein on phosphocellulose resin. No calmodulin-binding proteins or calmodulin-dependent kinase activity was observed in association with tubulin prepared after phosphocellulose chromatography (Figure 3). These data demonstrated that alpha and beta tubulin did not account for the calmodulin-binding proteins and calmodulin kinase activity associated with microtubule preparations. However, both the calmodulin kinase activity and calmodulin-binding proteins adhered to the phosphocellulose resin along with MAP-2 and tau

proteins. Thus, the kinase activity and the calmodulin-binding proteins could be removed from microtubule fractions along with the other microtubule associated proteins by phosphocellulose resin chromatography.

These results indicated that the two major calmodulin-binding proteins in microtubule fractions may represent the subunits of a calmodulin kinase and suggested that this kinase might be identical to a previously purified and well characterized calmodulin-dependent kinase which phosphorylates tubulin and microtubule-associated protein 2 (MAP-2) as major substrates (CaM Kinase II). Thus, we could determine by several structural and functional criteria if the two calmodulin-binding proteins in microtubule fractions represented the subunits of CaM Kinase II. The purified calmodulin-dependent kinase is composed of two calmodulin-binding subunits, designated rho and sigma, which like the calmodulin-binding proteins in microtubule preparations have molecular weights of 52,000 and 63,000 daltons, respectively, and isoelectric points near neutrality (Figure 1B). The calmodulin-binding proteins in microtubule preparations comigrated with the rho and sigma subunits of this calmodulin-dependent kinase on both one-dimensional and two-dimensional gels (Figure 1C).

When endogenous calcium-calmodulin-dependent microtubule protein phosphorylation was resolved in two-dimensional gel electrophoresis (Figure 4), phosphorylation of alpha and beta tubulin, MAP-2 and two proteins focusing near neutrality with molecular weights of 52,000 and 63,000 daltons was observed. These latter two phosphoproteins comigrated with both the calmodulin-binding proteins in microtubule preparations and the autophosphorylating subunits of purified calmodulin-dependent kinase (Figure 4B, C). In order to further compare these comigrating phosphoproteins in microtubule fractions with the autophosphorylating rho and sigma kinase subunits, the phosphoprotein spots from both purified calmodulin-dependent kinase and microtubule fractions were excised from two-dimensional gels, digested to completion with trypsin and tryptic phosphopeptides were resolved on two-dimensional thin-

layer electrophoresis/chromatography. The autophosphorylated rho (Figure 5A) and sigma (Figure 5B) subunits of purified calmodulin kinase display distinct phosphopeptide maps. the comigrating phosphoproteins observed in microtubule preparations (Figure 5C, D) demonstrated identical phosphopeptide patterns by comigration analysis (Figure 5E, F).

A number of parameters for calmodulin-dependent phosphorylation in microtubule preparations also demonstrated that the endogenous MT kinase functioned identically to the purified calmodulin-dependent kinase. As is the case with purified kinase, MAP-2 was a better substrate for calmodulin-dependent phosphorylation that tubulin. Also as with the previously reported values for purified kinase, the concentrations of calmodulin and ATP required to produce half-maximal activation of microtubule-associated calmodulin-dependent tubulin kinase were 50 nM and 7 uM, respectively. Finally, the purified calmodulin-dependent kinase demonstrates a characteristic pattern for phosphoamino acid phosphorylation on alpha and beta tubulin. As in the case of the purified kinase calmodulin-dependent phosphorylation in microtubule preparations on alpha tubulin occurred only on serine residues, while phosphorylation on beta tubulin occurred 60% on threonine and 40% on serine residues. Thus, by a number of functional criteria, the calmodulin-dependent kinase activity associated with microtubule preparations was identical to the purified calmodulin kinase. All of the data above indicated that the calmodulin-dependent kinase associated with microtubule preparations is CaM Kinase II.

These results identify two specific calmodulin-binding proteins associate with microtubule preparations and demonstrated that these calmodulin-binding proteins are the subunits of a calmodulin-dependent protein kinase system. While it is possible that other minor calmodulin-binding proteins may exist or might not be detectable by the techniques employed, any undetected calmodulin-binding proteins are likely to be minor components. thus, the subunits of calmodulin-dependent kinase are the major calmodulin-binding proteins associated with

microtubules and are likely candidates mediating some of the well-documented effects of calcium/calmodulin on microtubules. The data suggest and important role for CaM Kinase II in the regulation of microtubule and cytoskeletal dynamics.

3.   ASSOCIATION OF CaM KINASE II WITH TUBULIN

The isolation from brain cytosol of a complex containing alpha and beta-tubulin and the CaM Kinase II subunits was undertaken, demonstrating the association of a calmodulin dependent kinase with its endogenous substrate. Much of this work is now accepted for publication (Appendix).

Tubulin-CaM Kinase Complex

Rat brain cytosol was chromatographed on DEAE-cellulose and was eluted with 160 mM NaCl and 500 mM NaCl. All of the cytosolic tubulin, and significant calcium-calmodulin tubulin kinase activity was recovered in the 500 mM NaCl fraction. DEAE isolated tubulin kinase activity was chromatographed on a Sephacryl S-300 column and all of the tubulin kinase activity eluted as a single high molecular weight peak (Figure 6). Rechromatography of this peak on either Fractogel TSK HW-65 or Sephacryl S-300 yielded only a single high molecular weight peak, demonstrating the integrity of the complex. The protein staining pattern revealed both tubulin and also the CaM Kinase II subunits. The Fractogel-isolated complex demonstrated calcium-calmodulin dependent tubulin kinase activity and phosphorylation of proteins with molecular weights and pI's identical to the rho and sigma subunits of CaM Kinase II. As with the CaM Kinase II enzyme, the tubulin-enzyme complex phosphorylated beta-tubulin equally on both serine and threonine residues.

In the presence of chelator and 200 mM NaCl, the CaM Kinase-like kinase could be disassociated from tubulin. Passage over DEAE-cellulose then yielded kinase activity in the

void volume while tubulin adhered to the resin. The kinase activity that passed through the column adhered to calmodulin affinity resin. The kinase that eluted from the resin with chelator contained two peptide species of 52,000 and 63,000 daltons which were identical to the rho and sigma subunits of CaM Kinase II based on 2-dimensional tryptic peptide mapping. The results suggest a tight association between CaM Kinase II and tubulin in brain cytoplasm.

4.   <u>ASSOCIATION OF CaM KINASE II WITH MICROTUBULE PREPARATIONS AND ELECTRONMICROSCOPIC ANTIBODY LOCALIZATION OF CaM KINASE II ON MICROTUBULES</u>

Experiments in this research project studied the role of the tubulin-CaM Kinase II complex in MT polymerization. Microtubules polymerized by multiple cycles from the purified tubulin-CaM Kinase II complex manifested endogenous CaM Kinase II activity, indicating that CaM Kinase II is "truly" a microtubule associated protein. This result has been further confirmed by CaM Kinase II antibody studies on these preparations (Figure 7). Employing monoclonal antibodies to MAP-2, we have developed the ability to obtain high resolution EM antibody localization on negatively stained grids using colloidal gold-STAPH-A as an electrondense marker (Figure 7). Figure 7 demonstrates the binding of MAP-2 antibody to MT associated proteins (Figure 7B). The characteristic binding of polyclonal CaM Kinase II antibody to MT is also shown (Figure 7C). CaM Kinase II antibody was found to selectively decorate microtubules (Figure 7D). Furthermore, MT made from isolated tubulin devoid of CaM Kinase II, did not decorate with the CaM Kinase II antibody (Figure 7F). These preliminary results indicate that CaM Kinase II is associated with MAP-2 on MT sidearms, since the MAP-2 and CaM Kinase II antibody had similar localization patterns. Tau protein preparations also showed binding to the CaM Kinase II antibody and tau proteins could be immunoprecipitated with this antibody. The functional status of this kinase was further

investigated by comparison with the microtubule associated tau proteins which bind calmodulin. Our preliminary results indicate that the tau protein was homologous with the rho subunit of CaM Kinase II by peptide mapping. Thus, CaM Kinase II may represent an intrinsic calcium-calmodulin dependent regulatory system in the tau complex.

The distribution of CaM Kinase II with MTs was also investigated. MTs were prepared by three cycles of polymerization-depolymerization and studied for calcium-calmodulin kinase activity. We found that this MT fraction contained significant calmodulin kinase activity for tubulin and MAP-2 and also contained the two major calmodulin binding peptides described above. Preparation of tau proteins from this MT fraction also contained calmodulin kinase activity. Initial comparisons between CaM Kinase II and tau proteins indicate that they may be homologous proteins. Antibodies to tau obtained from Dr. N. Kirshner have also been found to react with CaM Kinase II in preliminary studies. Experiments will be initiated in this proposal to isolate CaM Kinase II and tau, and to carefully compare these preparations both structurally and functionally. Our preliminary results suggest that some of the tau proteins may represent a calmodulin kinase system associated with tubulin and MT preparations.

5. CaM KINASE II PHOSPHORYLATES MAP-2 AND TUBULIN ON DISTINCT PEPTIDE AND AMINO ACID RESIDUES

Recent studies in this grant period have demonstrated that CaM Kinase II phosphorylated tubulin and MAP-2 on specific sites, distinct from the sites of phosphorylation of these proteins by cyclic AMP kinases or other calcium-calmodulin kinases. Employing peptide mapping techniques and amino acid analysis of isolated peptides, we are now able to identify specific amino acid phosphorylation sited on tubulin and MAP-2 as "specific signatures of Cam Kinase II phosphorylation." These results will provide a powerful tool to investigate

the in vivo phosphorylation of tubulin and MAP-2 by CaM Kinase II in the intact synaptosome and neuronal culture systems described in this proposal.

6. CHARACTERIZED THE EFFECTS OF HYDRAZINE ON CaM KINASE II

Studies in this research effort have demonstrated that hydrazine alters the endogenous calcium stimulated phosphorylation of specific proteins in brain membrane. These results extended the studies in our previous grant that looked at whole brain homogenate phosphorylation. The effective amounts of protein substrates and concentrations of ATP were also determined. Optimal conditions were obtained for the substrates and a number of experiments were initiated to further characterize the effects of hydrazine on protein phosphorylation. Hydrazine inhibited calcium calmodulin dependent protein phosphorylation.

The effects of hydrazine on protein phosphorylation were studied over a wide range of hydrazine concentrations. As described in our previous experiments, hydrazine concentrations from 1×10^{-3} to 5×10^{-2} produced inhibition of membrane calcium calmodulin dependent protein phosphorylation. The half maximal concentration for inhibit was 1×10^{-2} molar.

In addition to inhibit of calmodulin dependent membrane phosphorylation, hydrazine also produced a stimulatory effect on membrane phosphorylation at lower concentrations. Hydrazine concentrations between 5×10^{-5} and 8×10^{-4} had a stimulatory effect on membrane calmodulin kinase activity. The maximal stimulatory effect was found at 4×10^{-4} molar. This stimulation accounted for a maximal increase of enzyme activity of approximately 50%.

In addition to these baseline studies, the effects of hydrazine on protein phosphorylation were studied over a wide range of pH. Hydrazine in inhibitory concentrations did not alter the pH optimum of the enzyme. In the excitatory kinase range, hydrazine also did not alter enzyme pH optimums. The time course of phosphorylation of specific membrane proteins was observed in the presence and absence of hydrazine. Hydrazine only effected the early phase

of kinase activation. No effect on long term phosphorylation was noted. In addition, we studied the effect of hydrazine on kinase activity in the presence of added 1 mM cold ATP. These conditions essentially block kinase activity and allow phosphatase activity to be quantitated. Hydrazine had no significant effect on phosphatase activity. In addition, in the presence of zinc, phosphatase activity was 90% inhibited and kinase was active. Under these conditions, we saw a clear inhibition of kinase activity by hydrazine. These results indicate that the inhibitory effect of hydrazine was directly on the calmodulin dependent kinase.

The inhibitory effects of hydrazine were compared to those effects of phenytoin on brain protein phosphorylation in membrane. Hydrazine inhibited calcium stimulated protein phosphorylation in the same manner as phenytoin. These results suggest that this drug, like phenytoin, may inhibit the effects of calmodulin in mediating calcium stimulated protein phosphorylation. At lower concentrations of hydrazine, the stimulatory effect on the kinase was also further studied. It appeared that this was a direct effect on the kinase and not an inhibition of phosphatase activity.

These studies indicate that the endogenous calcium calmodulin membrane kinase system can both be activated and inhibited by hydrazine at different concentrations. Very toxic concentrations of hydrazine can cause inhibition of this endogenous enzyme activity. However, at lower toxic concentrations the kinase can be activated and could account for stimulatory effects on the nervous system by exacerbating the calcium second messenger signal. Further studies are needed with more purified kinase.

7. EFFECTS OF HYDRAZINE ON CaM KINASE II ACTIVITY

The effects of hydrazine were tested on purified CaM Kinase II autophosphorylation and phosphorylation of synapsin I and microtubule associated protein two substrates. Hydrazine inhibited endogenous CaM Kinase II autophosphorylation and MAP-2 and Synapsin 1

phosphorylation in high concentrations. Half maximal inhibition of CaM Kinase II activity was found at approximately 1×10^{-2} molar. CaM Kinase II activity could be totally inhibited at 9×10^{-2} molar.

At lower hydrazine concentrations we did not observe CaM Kinase II stimulation with lower concentrations of hydrazine. These results suggested that the effect of hydrazine in stimulating CaM kinase activity in the membrane may have been related to the hydrophobic effects of this compound on membrane fluidity. It is possible that this could have effected kinase accessibility to calmodulin or ATP and therefore cause excitation at lower concentrations. However, with the purified enzyme we did not see a direct stimulatory effect on the kinase with hydrazines.

8. STUDY THE EFFECTS OF CaM KINASE II ON MICROTUBULE POLYMERIZATION

Studies in this research period have shown that CaM Kinase II co-purifies with microtubules and neurofilaments. The results of these studies have been published and are included in our publication list. This work was very important in establishing the role of calcium in cytoskeletal function. Our results indicate that CaM Kinase II is a microtubule associated protein in cold-stable microtubules and neurofilaments. This kinase could be activated by calcium and calmodulin in the microtubule and neurofilament preparations. These results are presented above in more detail in the actual studies that were done. At this point we wanted to determine if endogenous CaM Kinase II associated with cold stable microtubules could effect microtubule polymerization. These studies were initiated in this grant and provided important information on how calcium may regulate cytoskeletal function.

Cold stable microtubules that contained endogenous CaM Kinase II activity were incubated in the presence of calcium and calmodulin. In these preparations, calcium and calmodulin had a very potent effect, causing depolymerization of the microtubule structure.

The concentrations of calcium and calmodulin that achieved this depolymerization were in the physiological range. The half maximal concentration of calcium was approximately 10 uM and calmodulin levels were also in the micromolar range. These results indicate that physiological calcium and calmodulin levels can depolymerize microtubules under conditions where they would activate calmodulin kinase activity. These studies strongly suggest that calcium and calmodulin depolymerize microtubules through activation of Calmodulin Kinase II. However, we cannot rule out from these studies that there were other endogenous calcium binding proteins associated with the microtubules. To test this possibility, we used more purified microtubule components to study the effect of CaM Kinase II on microtubule polymerization.

Highly purified tubulin devoid of microtubule associated proteins and CaM Kinase II was prepared. This tubulin would not easily polymerize under standard conditions in the absence of MAP 2 or other microtubule associated proteins. We were able to prepare a MAP 2-CaM Kinase II complex that was added to the purified, highly enriched tubulin fraction. This MAP 2-CaM Kinase complex was able to support polymerization of tubulin into microtubules. Thus, these microtubules contained primarily tubulin and highly enriched CaM Kinase II and MAP 2. No other calmodulin binding proteins were detected by multiple procedures. These microtubules were not depolymerized by micromolar concentrations of calcium alone. In the presence of physiological calcium and calmodulin concentrations, these microtubules did depolymerize.

These results strongly indicate that calcium and calmodulin are acting by activation of CaM Kinase II. Preliminary experiments with microtubules that were polymerized with MAP 2 and tubulin but lacking CaM Kinase II showed that these microtubules were resistant to physiological calcium and calmodulin concentrations. These microtubules only depolymerized at concentrations of calcium in the millimolar range.

9. **CORRELATED EFFECTS OF HYDRAZINE ON CaM KINASE II AND MICROTUBULE POLYMERIZATION**

The microtubule experiments presented above were carried out in the presence or absence of hydrazine. In millimolar concentrations of hydrazine, we could successfully block CaM Kinase II activation in microtubule preparations. Under these conditions, calcium and calmodulin did not actively depolymerize microtubules. Thus hydrazine prevented microtubule depolymerization under these conditions. These results indicate that hydrazine inhibition of CaM Kinase II could modulate the effects of calcium on cytoskeletal dynamics. The role of these effects in mediating some of the chronic toxic effects of hydrazine on peripheral nerves and the nervous system must be considered. This could be a physiological mechanism by which hydrazine may effect cytoskeletal structure and neurofunction.

10. **INVESTIGATED THE EFFECTS OF PHARMACOLOGICAL AGENTS ON MODULATING EFFECTS OF HYDRAZINE ON CaM KINASE II**

We studied the effects of numerous anticonvulsant and convulsant drugs on the inhibitory effects of hydrazine on CaM Kinase II. We did not find any drugs that clearly blocked the hydrazine inhibition of CaM Kinase II. We concluded from this that CaM Kinase II is probably directly interacting with the enzyme and inhibiting kinase activity. This kinase inhibition could not be overcome with ATP or increasing calmodulin concentrations. This suggested that hydrazine is not competing for the ATP or calmodulin binding site of the enzyme. Further studies are needed, however, to look at drugs that could block the hydrazine effect without causing toxic effects in man. Further study of these agents are currently being investigated.
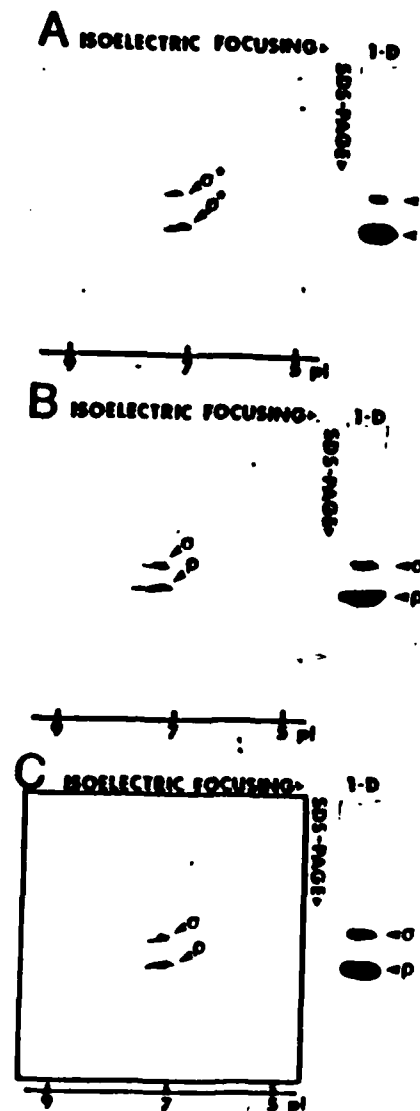
Figure 1. Calmodulin-binding proteins in microtubule protein
preparations and purified calmodulin-dependent kinase. A.
Autoradiographs [125I]-calmodulin in microtubule preparations in
one-dimensional SDS-PAGE (1-D) and two-dimensional gels. Both
show the presence of two calmodulin-binding proteins with
approximate molecular weights of 52,000 and 63,000 daltons, both
with isoelectric points near neutrality. All detectable
calmodulin-binding was abolished in the presence of 2 mM EGTA.
B. Calmodulin-binding subunits of purified calmodulin-dependent
kinase visualized in one-dimensional and two-dimensional gels
show two subunits of 52,000 (ρ) and 63,000 (σ) daltons, both
with isoelectric points near neutrality. Comigration analysis
illustrated in C revealed that the calmodulin-binding proteins in
microtubule preparations demonstrated identical migration on one-
dimensional and two-dimensional gels to the σ and ρ subunits of
purified calmodulin kinase. One-dimensional gels were over
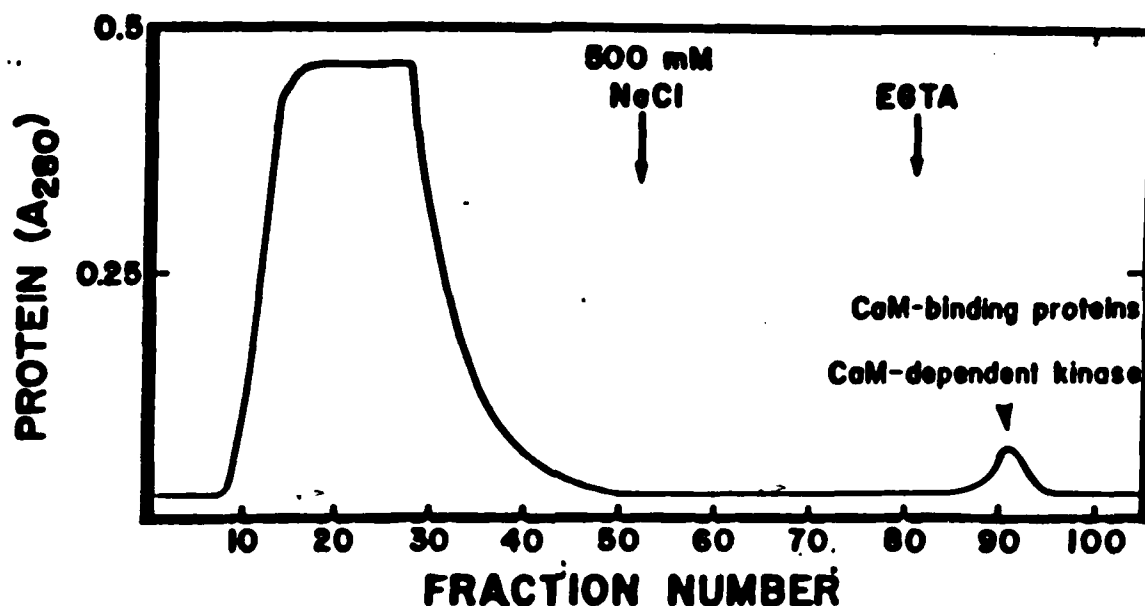exposed to demonstrate the absence of other detectable bands.

Figure 2. Calmodulin affinity chromatography of microtubule protein. Twice-cycled microtubule protein (20 mg) was loaded onto calmodulin affinity resin. The column was then sequentially eluted with equilibration buffer containing 500 mM NaCl, and then buffer containing 2 mM EGTA. Less than 2.5% of the applied protein eluted with the chelator fraction which contained 52,000 and 63,000 dalton calmodulin-binding proteins and calmodulin-dependent kinase activity.
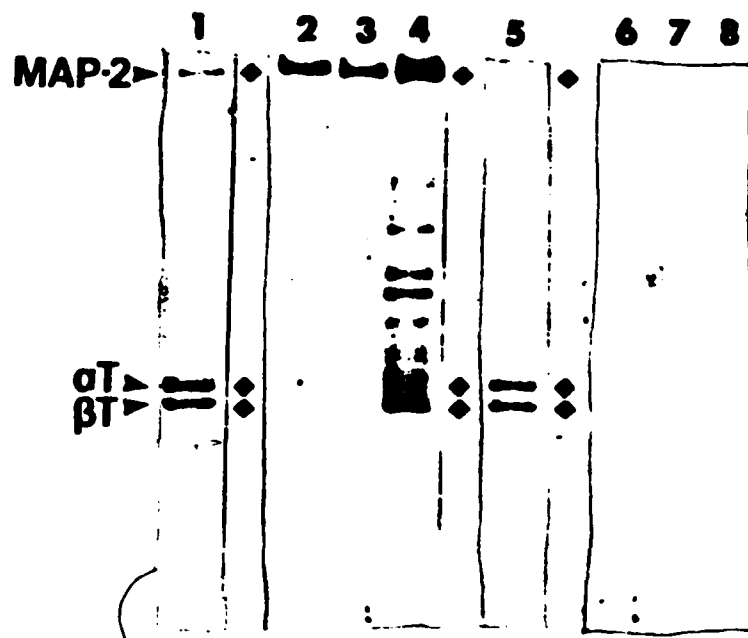
Figure 3. Calmodulin-dependent phosphorylation in microtubule preparations. Lane 1: Coomassie blue staining pattern of thrice-cycled microtubule preparations showing alpha tubulin (αT), beta tubulin (βT) and MAP-2 as major protein staining species. Microtubule preparations were phosphorylated in the presence of Mg2+ only (Lane 2), Mg2+ and Ca2+ (Lane 3) and Mg2+, Ca2+, calmodulin (CaM; Lane 4). Lane 5 shows Coomassie blue staining for homogeneous tubulin prepared by phosphocellulose chromatography. When homogeneous tubulin was phosphorylated in the presence of Mg2+ only (Lane 6), Mg2+ and Ca2+ (Lane 7), and Mg2+, Ca2+ and calmodulin (Lane 8) no phosphorylation was observed.
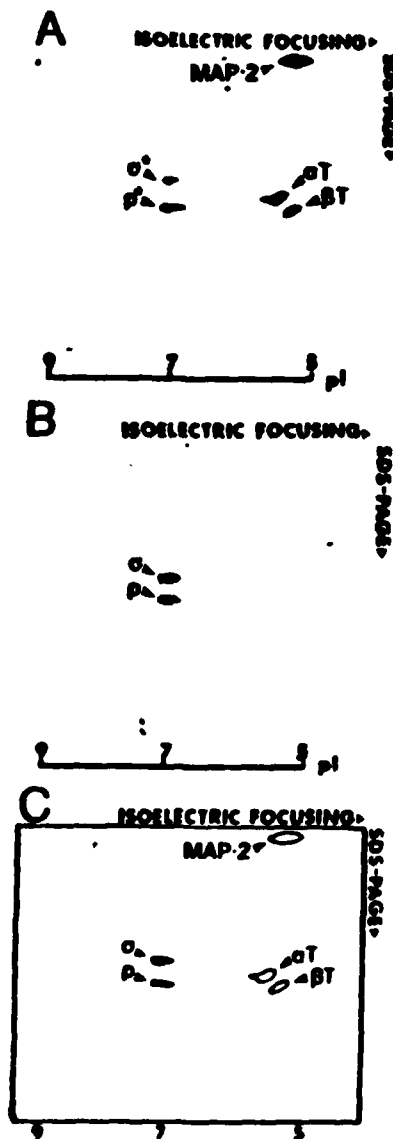
Figure 4. Resolution of calmodulin-dependent phosphorylation in microtubule preparations in two dimensions. A. Resolution of calmodulin-dependent phosphorylaiton in microtubule preparations displays the phosphorylation of alpha tubulin (αT), beta tubulin (βT) and MAP-2, as well as the phosphorylation of two protein (ρ and σ^*) with molecular weights and isoelectric points similar to the purified calmodulin-dependent kinase. B. Resolution of the autophosphorylated subunits of purified calmodulin-dependent kinase in two dimensions shows the typical pattern for the   and subunits.   Comigration analysis illustrated in C revealed that the two neutral phosphoprotein species in microtubule preparations comigrated with the ρ and σ subunits of purified kinase. The locations of alpha and beta tubulin and MAP-2 are also shown.
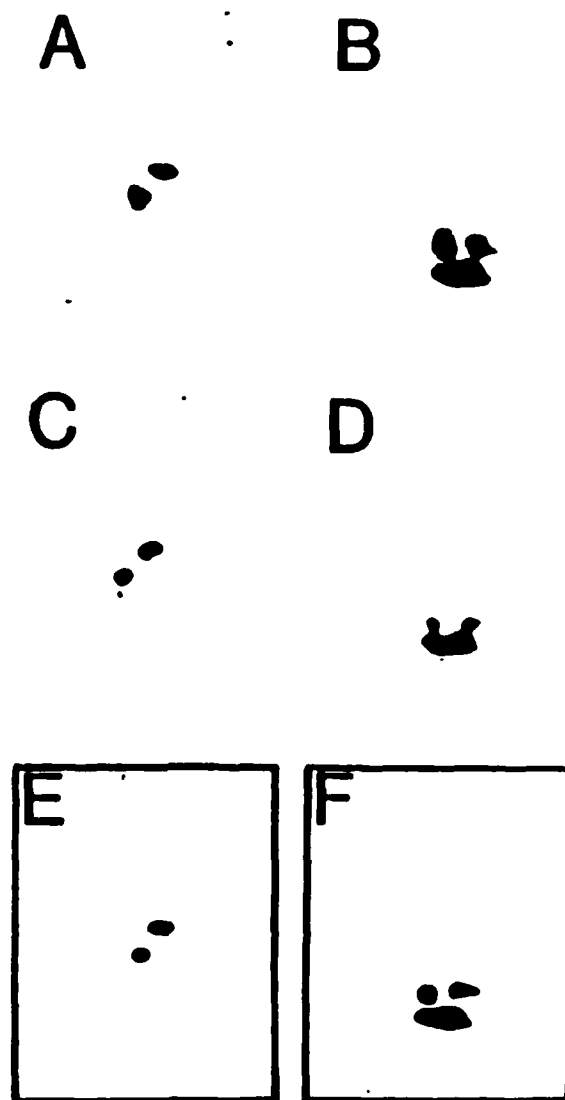
Figure 5. Two-dimensional tryptic phosphopeptide mapping of microtubule phosphoprotein spots comigrating with ρ and σ. A. Phosphopeptide map for the autophosphorylated ρ subunit of purified calmodulin-dependent kinase. B. Phosphopeptide map for autophosphorylated σ subunit of purified calmodulin kinase. C. Phosphopeptide map of the 52,000 dalton neutral phosphoprotein in microtubule preparations (ρ^* in Figure 4). D. Phosphopeptide map of the 63,000 dalton neutral phosphoprotein in microtubule preparations (σ^* in Figure 4). E. Comigration of A and C revealed indistinguishable maps. F. Comigration of B and D reveal indistinguishable maps.
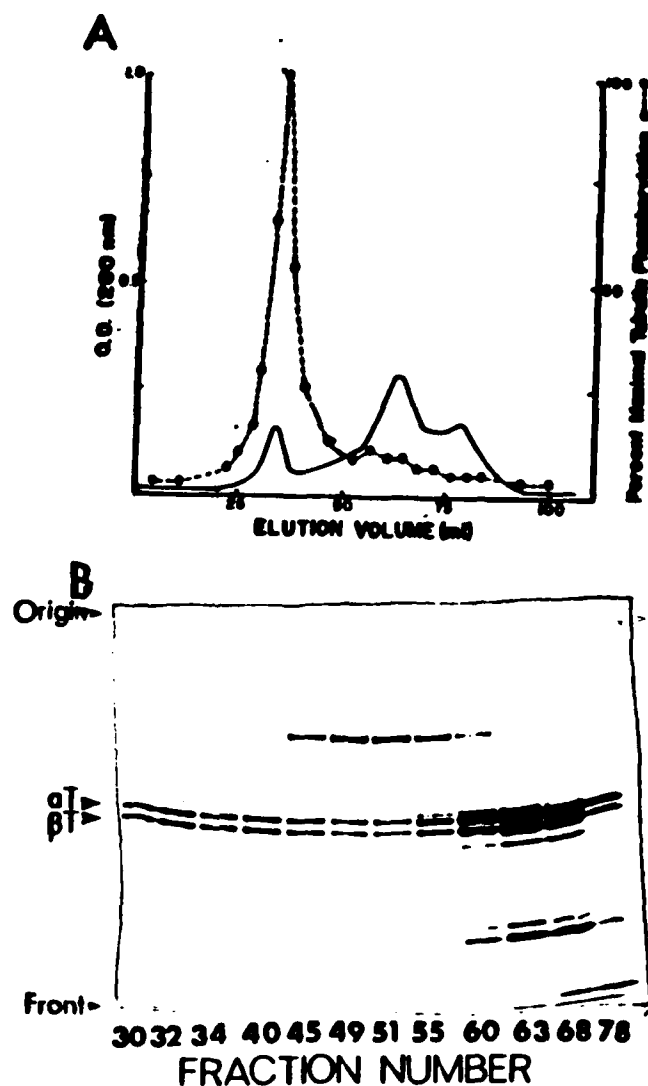
A

B

FRACTION NUMBER

FIGURE 6. Resolution of the 500 mM NaCl elution from DEAE cellulose
on Sephacryl S-300 gel chromatography resin. A. The 500 mM NaCl
elution from DEAE resin was applied to Sephacryl S-300 column (1.6 X
80 cm) and eluted with 10 mM Pipes, pH 6.9, 0.1 mM MgCl2, 1 mM 2-
mercaptoethanol, 0.3 mM PMSF. Elution of protein was monitored at 280
nm (——). One ml fractions were collected and assayed for
endogenous calmodulin dependent tubulin kinase activity (●--●) under
standard conditions. Tubulin kinase activity eluted in the void
volume of the column. B. SDS-PAGE resolution of fractions eluted from
Sephacryl S-300. Fractions 30, 32 and 34 correspond to the peak of
endogenous tubulin kinase activity and demonstrated alpha and beta
tubulin as the major protein components with a few minor staining
polypeptides. The majority of the tubulin and other proteins eluted
from DEAE chromatographed in the major included peaks (Fractions 49
through 78).

Figure 7. Antibody decoration of microtubules by MAP-2 monoclonal
antibodies (A,B) and polyclonal TACK antibody (C-F). The antibody
labelling procedure was modified from F.J. Roll, J.A. Madri,
J. Albert, and N. Furthmayer (J. Cell Biol. 85: 597-616, 1980).
Thrice cycled MT preparations were lightly fixed with glutaraldehyde
and adsorbed on to glow discharged, carbon coated grids. Each grid
was then incubated with the specific antibody or appropriate control
antibody samples. Following extensive washing, the antibody, bound
to the grid was detected with colloidal gold labelled STAPH-A. The
gold particles can be seen as black dots on the photomicrographs.
This figure is presented to demonstrate the use of this technique in
studying the structural association of specific proteins with
microtubules. The specific MAP-2 monoclonal (7B) and control sera
(7A) demonstrate the specific labelling of the MT sidearms with
MAP-2 antibody. These results confirm previous observations.
Figure 7C presents the specific labelling with TACK antisera to
compare it to MAP-2 labelling. TACK antibodies decorated the
sidearms of the MT in a similar pattern to the MAP-2 antibody.
Figure 7D-F presents the specificity of the TACK antibody. Control
preimmune sera (7E) showed no significant labelling in comparison to
TACK antibody (7D). MT prepared from PC-tubulin and purified MAP-2
also did not decorate with TACK antibody (7F). These MTs did not
contain TACK by biochemical criteria and also did not decorate with
TACK antibody. We will employ this and other EM antibody labelling
procedures to determine the localization of MAP-2, TACK, α and β-
tubulin on MT structures.
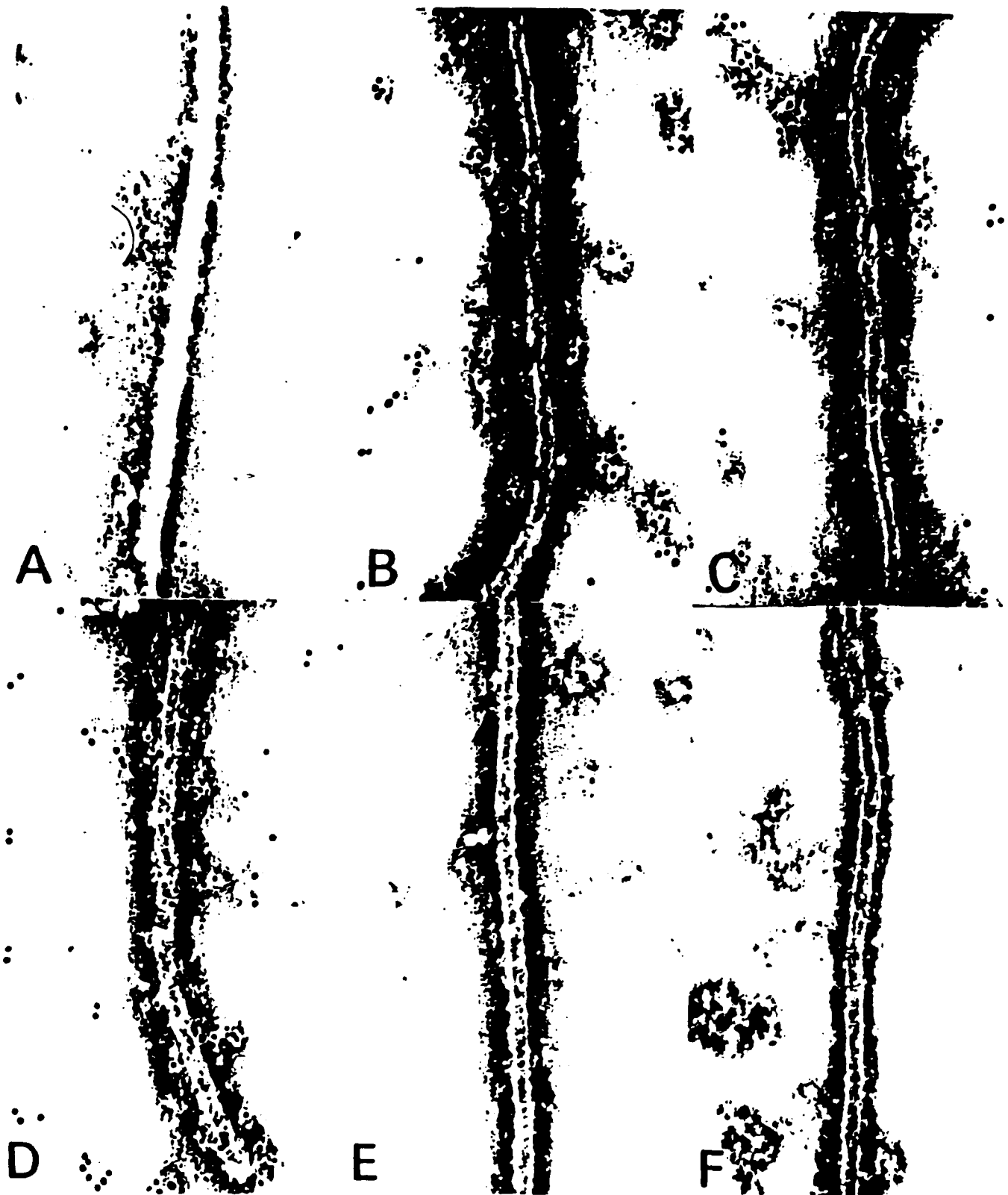
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